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Reduction of Sample Matrix Effects – The Analysis of Benzimidazole Residues in Serum by Immunobiosensor

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Regulatory authorities, the food industry and the consumer demand reliable determination of chemical contaminants present in foods. A relatively new analytical technique that addresses this need is an immunobiosensor based on surface plasmon resonance (SPR) measurements. Although a range of tests have been developed to measure residues in milk, meat, animal bile and honey, a considerable problem has been encountered with both serum and plasma samples. The high degree of non-specific binding of some sample components can lead to loss of assay robustness, increased rates of false positives and general loss of assay sensitivity. In this paper we describe a straightforward precipitation technique to remove interfering substances from serum samples to be analysed for veterinary anthelmintics by SPR. This technique enabled development of an assay to detect a wide range of benzimidazole residues in serum samples by immunobiosensor. The limit of quantification was below 5 ng/ml and coefficients of variation were about 2%.

Keywords: *Biosensor, matrix effects, surface plasmon resonance, albendazole*

INTRODUCTION

Modern farming utilizes veterinary drugs to minimize the impact of disease on food animal production. At the same time, consumers increasingly demand animal products that are free from microbiological and chemical contamination. There are more than 3000 licensed veterinary drugs on the market, all with different effects and withdrawal periods (Baxter, 1999). One such group of compounds is the benzimidazole anthelmintics which are used to treat and prevent worm infestations in farm animals. The first highly efficient broad spectrum anthelmintic was thiabendazole (McKellar & Scott, 1990), but today more than 20

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benzimidazole derivatives are used in veterinary and human medicine. Benzimidazoles exert their anti-worm effects by selective binding to parasite tubulin, disrupting the tubulin-microtubule dynamic equilibrium and negatively affecting cell division (Lacey, 1990).

There are many different analytical methods used for screening of veterinary drugs in animal products. Agar diffusion (Currie *et al.*, 1998), enzyme immunoassay (Brandon *et al.*, 1994; Brandon *et al.*, 1998), thin-layer chromatography (Haagsma *et al.*, 1984) and liquid chromatography (Marti *et al.*, 1990; LeVan and Barnes, 1991; Domany and Kovacsics, 2000) are typical examples. More recently, biosensor assays have been shown to be highly efficient and reliable techniques for performing drug residue screening in biological samples (Baxter *et al.*, 2001). A wide range of different matrices are used in residue monitoring programmes (e.g., blood, solid tissues, and urine), and the matrix of choice is often dictated by the pharmacokinetic characteristics of a drug as well as the ease of obtaining the sample. Matrix effects, broadly defined as interference with the analytical technique by one or more components of the sample, may influence the preferred combination of sample and analytical technique (Crooks *et al.*, 1998a, Crooks *et al.*, 1998b, Baxter *et al.*, 1999). In general, matrix effects can lead to loss of assay robustness, loss of sensitivity and high levels of false positive and false negative results. Biosensor assays for drug residues based on surface plasmon resonance (SPR) measurements are subject to pronounced matrix effects when complex samples are analysed (Luong *et al.*, 1997). Non-specific interactions of sample components with the antibody and/or the biosensor chip surface appear to be involved.

The present study was undertaken to elucidate the nature of serum matrix effects in biosensor assays. This paper demonstrates a procedure to minimize matrix effects, resulting in a sensitive and precise technique for detecting albendazole residues in serum samples.

MATERIALS AND METHODS

Equipment

The optical biosensor used was a Biacore® 1000 (Biacore AB, Uppsala, Sweden), with Biacore® 1000 Control Software version 2.2. BlAevaluation version 3.0.2 was used for data handling.

Reagents and Chemicals

CM5 sensor chips (Research Grade), amine coupling kit and HBS-EP buffer (**HBS**)(0.01 M-HEPES buffer, 0.15 M NaCl, 3 mM-EDTA, 0.005% v/v, polysorbate 20, pH 7.4) were obtained from Biacore AB (Uppsala, Sweden). Bovine albumin and globulin were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). Ammonium sulphate was obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). The hapten, 5(6)-((carboxypentyl)thio)-2-((methoxycarbonyl)amino)-benzimidazole, was previously described (Brandon *et al.*, 1994). Filters were obtained from Millipore AB (Sundbyberg, Sweden). Samples of bovine serum were collected from a farm where the use of veterinary drugs is under strict government control.

Other buffers used in the study were: **SDB** (pH 7.4), 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, 2.7 mM-KCl, 500 mM-NaCl and 0.5% Tween 80; **PBS1** (pH 7.2), 40 mM-NaH_2PO_4, 16 mM-Na_2HPO_4 and 150 mM-NaCl; **PBS2** (pH 8.2), 4 mM-NaH_2PO_4, 16 mM-Na_2HPO_4 and 150 mM-NaCl.

Antibody Production

The antibody used in the assay was raised in a sheep immunised against the carboxy albendazole derivative, 5(6)-((carboxypentyl)thio)-2-((methoxycarbonyl)amino)-benzimidazole. This hapten was coupled to a protein carrier, human serum albumin, to make it immunogenic (Erlanger *et al.*, 1957). Prior to injection, the immunogen was emulsified with

Freund's complete adjuvant; subsequent booster injections contained Freund's incomplete adjuvant. During each immunization the sheep was injected intramuscularly at four sites. About 10 days after each booster injection a blood sample was taken to monitor the antibody titre by ELISA. An antiserum denoted as S48 was used in this study.

Immobilization of Albendazole Derivative to Sensor Chip Surface

The chip surface was activated by adding a mixture (1:1) of 50 mM-N-hydroxysuccinimide (NHS) and 0.2 M-l-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), converting the carboxy groups to reactive NHS-esters. Forty μl of ethylenediamine was added (pH 8.5) to introduce free amine groups. Remaining NHS esters were deactivated by treatment with 1 M-ethanolamine for 20 min to minimise interference with immobilization of hapten. To activate the carboxyl group of the hapten, NHS (2 mg) and EDC (5 mg) were mixed in 450 μl of 10 mM-sodium acetate (pH 4.5) and added with mixing to 2 mg of the carboxy albendazole derivative in 450 μl DMF. Fifty μl of this solution were flowed onto to the chip surface and incubated at 25°C for 2 h. When not in use, the chip was stored desiccated between 6 and 8°C. Before the chip was used it was extensively washed with HBS buffer and dried under a stream of nitrogen.

Biosensor Assay Conditions

All studies were conducted at 25°C, using a flow rate of 25 μl/min. Binding to the chip surface was indicated by changes in the SPR signal, expressed in arbitrary resonance units (RU). Antiserum S48 was diluted 1:1000 in HBS buffer and is referred to as 'antibody' below. Standard solutions of albendazole were prepared in HBS buffer. Three assay formats were tested to analyse matrix effects.

Format I. Bovine serum was mixed with HBS buffer, standard and antibody, and the solution was injected onto the coated chip for 2 min. The chip was regenerated by the injection of 15 μl of 25 mM-HCl followed by 20 μl of 180 mM-NaOH after each sample analysis. Increasing amounts of serum (50–200 μl/ml) were added to determine the cumulative matrix effect of sample components during analysis.

Format II. Bovine serum samples (100 μl/ml) were mixed with a range of buffers (HBS, SDB, PBS1 and PBS2), standard solutions and antibody. Each of the buffers was then injected over the chip surface and a similar injection was repeated immediately after the chip surface was regenerated.

Format III. Saturated ammonium sulphate (SAS) was employed to precipitate the proteins present in the bovine serum samples. In each autosampler vial, 85 μl SAS, 85 μl spiked serum sample and 30 μl HBS buffer were mixed. The mixture was vortexed twice for 5 sec and centrifuged for 10 min at 14,000 rpm. Finally, 20 μl of the supernatant was mixed with 180 μl antibody and analysed on the biosensor as above. Format III was characterised with regard to cross-reactivity, accuracy, detection limits and reproducibility.

Further Investigation of Matrix Effects

Three additional procedures were employed.

1. Solutions of immunoglobulin and albumin (30 mg/ml) were prepared in HBS buffer. These solutions were injected (2 min/sample), individually and combined, over the surface of the albendazole-derivatized chip.
2. Serum samples spiked with albenbazole were passed through 0.22 μm or 0.45 μm filters or filters with nominal size exclusions of 5 kDa, 10 kDa or 30 kDa. Filtrates were injected over the chip as previously described.
3. Dextran (1 mg ml/ml) was added to the HBS buffer solution. This buffer was used to dilute serum samples prior to injection over the derivatized chip.

RESULTS

The derivatised chip A single albendazole-immobilised chip was used throughout the three month study. The anti-albendazole antibody consistently bound to the chip and a steady baseline was achieved post-regeneration. It was concluded that the chip surface was highly stable under the described conditions for preparation, utilisation and storage.

Calibration curves Albendazole calibration curves were constructed in HBS-EP buffer. Figure 1 illustrates the direct analysis of serum. Pronounced matrix effects are indicated by the increase in relative response units, i.e. apparent binding of serum components onto the chip surface. Binding increased as the concentration of serum increased. An increase in coefficients of variation was observed at the higher levels of serum.

The use of alternate buffering systems to reduce the matrix effect had a limited success (Figure 2), but SDB buffer exhibited only about half the matrix effect compared to HBS-EP, presumably because of its higher ionic strength.

Figure 3 illustrates the results obtained using the SAS sample preparation procedure. The curves produced using this method were very similar to those achieved in the buffer system. Coefficients of variation were lower than those found in the direct serum assays. These results strongly indicate that non-specific binding and decreased precision of the assay are caused by one or more protein components of the serum which are removed by precipitation. The increased ionic strength due to the addition of ammonium sulphate probably contributes to the reduced non-specific binding, as was found with SDB buffer.

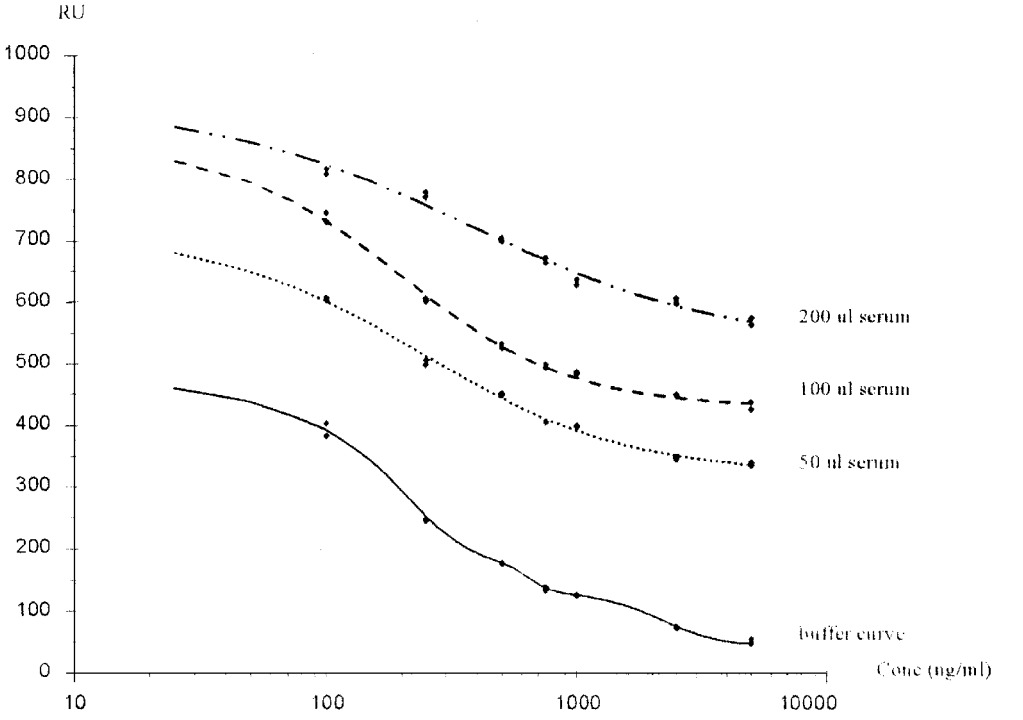

FIG. 1. Albendazole biosensor assay. Various volumes of control serum were added to the autosampler vials containing antibody and albendazole standards and subsequently injected into the biosensor. The solid line represents the assay conducted in the absence of serum.

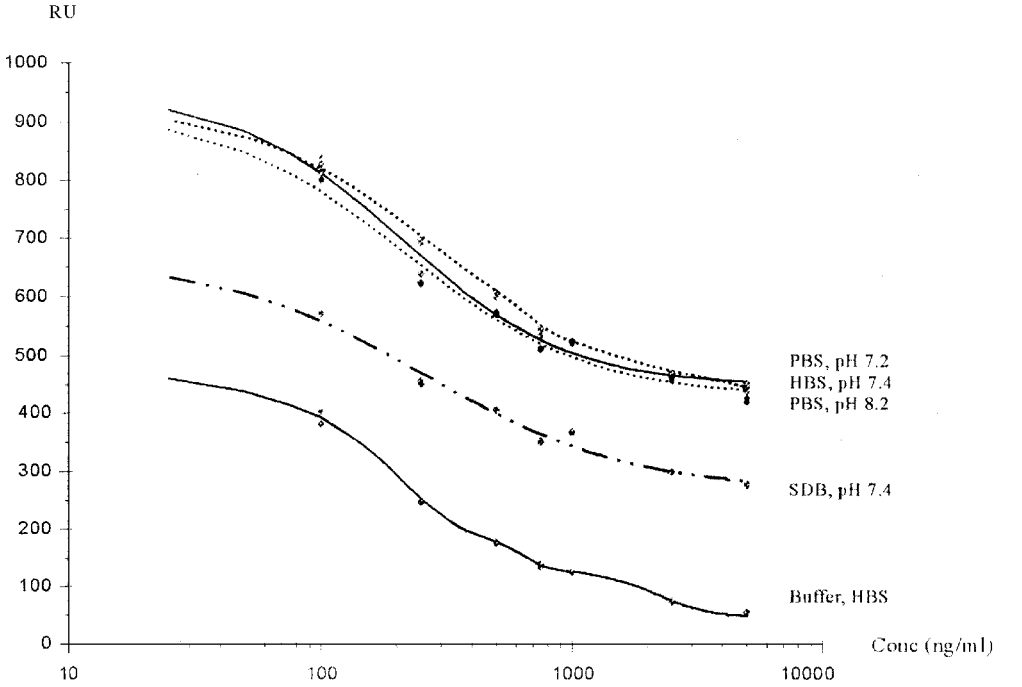

FIG. 2. Effect of buffering system on the matrix effect. Use of SDB resulted in a decrease in matrix effect by approximately 50%. The solid line represents the assay conducted without the addition of serum.

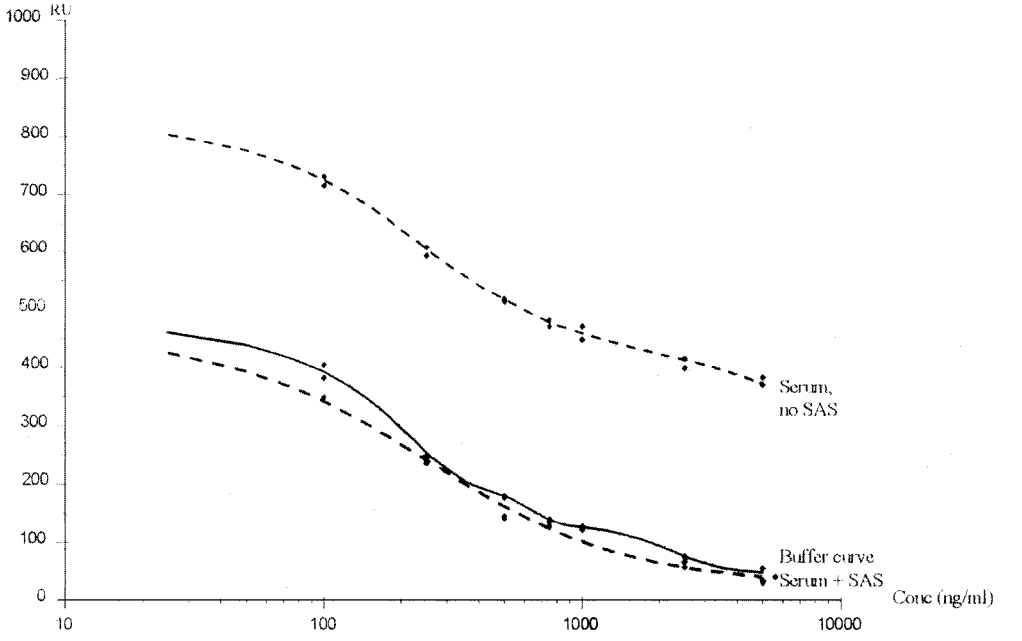

FIG. 3. Effect of SAS precipitation on matrix effect. Spiked serum samples were analysed for albendazole directly or after precipitation by SAS. The solid line represents the assay conducted in the absence of serum.

TABLE 1. Cross-reactivity profile of antiserum S48 determined as [IC50 (albendazole)/IC50 (test compound)] × 100

Benzimidazole	% Cross-reaction
Albendazole	100
Fenbendazole sulphone	89.6
Mebendazole	82.0
Flubendazole	75.2
Oxibendazole	74.1
Carboxy albendazole hapten	67.1

Antibody cross-reactivity The cross-reactivity profile of antibody S48 was determined by testing a range of benzimidazoles in the presence of serum matrix, following the procedure of Format III. The results are summarized in Table 1. The antibody displayed cross-reactivity ranging from 67–90% for the different benzimidazoles studied. Thus, the sheep antibody could be considered group-specific, as opposed to compound-specific. This conclusion was found in earlier studies which developed mouse monoclonal antibodies using the same hapten and in our previous study in a bovine liver matrix (Brandon *et al.*, 1994; Crooks *et al.*, 2000).

Cause of matrix effects Additional experiments were performed to determine the cause of the serum matrix effect. Injection of solutions of bovine serum globulins and albumins produced non-specific binding to the chip surface. Both proteins alone or in combination caused binding of about 150 resonance units.

The filtration of bovine serum through 0.22 μm or 0.45 μm filters did not significantly alter the matrix effect. However, ultrafiltration of the serum through filters with nominal exclusion limits of 5, 10 or 30 kDa appreciably lowered the levels of non-specific binding. When serum samples were spiked with albendazole and subsequently filtered, it appeared that much of the drug content of the sample was lost during the process. This may have been caused by weak binding of albendazole to a high molecular weight component in the serum or by binding of the drug to the filtration membranes.

The addition of dextran to the buffer had no effect on the serum matrix effect. We had hypothesised that free dextran in solution might compete for the binding of serum components with the dextran that is part of the chip surface and thus reduce the amount of non-specific binding to the chip surface. It therefore appears that binding sites on dextran are not responsible for the matrix effect of serum.

Assay validation (SAS precipitation method) The robustness of the SAS precipitation method was investigated by analysing a range of bovine serum samples. Ten serum samples, taken from different animals, were analysed following SAS precipitation. Each of the sera was spiked with increasing concentrations of albendazole (0 to 5000 ng/ml) and analysed as described above. The results are shown in Table 2. Only the highest spiking concentration used (5000 ng/ml) produced a CV in excess of 20%. This effect was due to the low relative responses measured at the high drug concentrations. The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated from assaying 20 blank bovine serum samples. The values obtained were 2.6 ng/ml (mean concentration plus three standard deviations) and 4.8 ng/ml (mean plus 6 standard deviations), respectively.

In order to investigate the precision of the method, three serum samples were spiked with 10, 50 and 250 ppb of albendazole and analysed six times. The average RU values and the CV were calculated (Table 3). The results showed excellent reproducibility (range 0.8 to 1.5%).

TABLE 2. Variation in responses measured during the injection of serum samples (n = 10) with increasing concentrations of albendazole present

Spiking conc. (ng/ml)	Mean response (RU)	CV (%)
0	525.2	5.4
100	416.0	6.5
500	153.3	10.8
1000	94.2	16.0
5000	32.9	36.2

TABLE 3. Determination of precision of analysis of serum samples spiked with albendazole at three concentrations.

Cycle number	10 ppb Ru	50 ppb Ru	250 ppb Ru
1	617.1	560.7	331.8
2	616.8	564.2	339.3
3	616.3	565.9	342.5
4	616.3	571.4	345.9
5	619.1	566.0	340.9
6	628.8	576.8	344.0
Mean (RU):	619.1	567.5	340.7
S. d. (RU):	4.9	5.7	4.9
CV (%):	0.8	1.0	1.5

DISCUSSION

This is the first description of a sensor-based assay for benzimidazole residues in serum. The broad specificity of the antibody produced and the rapidity of the sample preparation technique and analysis make it a very attractive alternative to multi-benzimidazole assays such as HPLC.

The underlying causes of matrix effects encountered with serum samples seem related to non-specific interactions with the derivatised chip surface, rather than to interaction with the specific antibody. Thus, increases in RU are noted even in the absence of specific antibody. Attempts to reduce the matrix effect by changing the buffer or fractionating the sample by filtration were only partially successful. However, the precipitation of serum proteins prior to sample analysis was completely successful in removing non-specific binding and in improving the reproducibility of the assay. The working range, shape and precision of the albendazole standard curves in the presence of serum supernatant were indistinguishable from the buffer counterpart.

As a sample preparation method for routine use, the procedure utilised is both simple and quick. There are, however, a number of important limitations to this method which must be considered. It cannot be assumed that the precipitation method will work for the determination of other drug residues in sera. The binding of drugs to serum components is a major factor influencing the viability of this procedure. There are a large number of serum proteins with the function of transporting low molecular weight. It may be that when such proteins are precipitated any drugs bound will also be removed from solution components (Olson and Christ, 1996). A second limiting factor in the precipitation technique relates to the ionic strength of the solution to be injected over the sensor surface. It is known that some antibodies cannot function at high ionic strengths. In the present case there appeared to be no effect on the polyclonal antibody population in the sheep serum.

Serum is only one of the matrices which are used in drug residue analysis. Similar deleterious matrix effects are known to exist with samples such as urine, fat and solid tissues. A wide array of different matrix components may be responsible for these effects. For biosensor technology to become accepted as a means of performing routine screening analysis, these components must be identified. The SAS precipitation method developed to allow the determination of albendazole residues in serum has many advantageous features. The sample preparation is simple and fast and the analytical procedure, robust, sufficiently sensitive, and highly reproducible. These attributes make the possibility of sensor-based analysis inviting to analysts who require fast and reliable screening determination of drug residues in biological samples. It may be that this procedure can be adapted for the analysis of drug residues in more complex samples such as meat and offal.

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